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Investigation of tetracycline resistance genes in *Escherichia coli* isolates from broiler chickens during a rearing period in Iran

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ABSTRACT

The tetracycline resistance of *E. coli* isolates (n = 300) from broiler chickens was investigated in three stages of the rearing period (one-day-old chicks, thirty-day-old chickens and one day before slaughter). Tetracycline resistance genes (*tet*(A), *tet*(M), *tet*(O) and *tet*(S)) were investigated among 120 tetracycline resistant *E. coli* isolates. Tetracycline resistance at the three stages of sampling was 67, 90 and 94%, respectively. Of the 120 tetracycline resistant *E. coli* isolates, 68 (59%) carried the *tet*(A) resistance gene. The *tet*(A) resistance gene was present in 32.5% (13/40) of *E. coli* isolated from one-day-old chicks, 65% (26/40) of *E. coli* isolated from thirty-day-old chickens and 72.5% (29/40) of *E. coli* isolated from the chickens on the day before slaughter. None of the tested isolates contained *tet*(M), *tet*(O) or *tet*(S). Tetracycline resistance was relatively high in *E. coli* isolated from one-day-old chicks, suggesting a high prevalence of tetracycline resistance in the preliminary gut flora of these broiler chicks. Significant increases in the resistance rate of *E. coli* isolates were found during the 2nd and 3rd rearing period. *Tet*(A) was the only detected *tet* resistance gene in these *E. coli* isolates. The rise of the *tet*(A) resistance gene during the rearing of broilers is alarming because this plasmid mediated *tet* gene can be transmitted to other pathogenic and commensal bacteria in the poultry industry.

Key word: *Escherichia coli*, tetracycline resistance, *tet*(A), broiler chickens

Introduction

Escherichia coli is one of the most important bacterial pathogen in broiler chickens, because this genus of the enterobacteriaceae family is the main causative agent of cellulitis, septicemia, and airsacculitis in poultry (SMITH et al., 2007). *E. coli* is present in

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the normal microflora of the intestinal tract of poultry. These intestinal serotypes could act as a reservoir of antimicrobial resistance factors for extra-intestinal isolates (ANDERSON et al., 2014; NÓGRÁDY et al., 2006). Moreover, there is evidence of the transmission of resistant clones and resistance plasmids of *E. coli* from poultry to humans (VAN DEN BOGAARD et al., 2001).

Due to its low cost, efficacy, and lack of side effects, the tetracycline family is one of the most commonly used antibiotics in the poultry industry (CHOPRA and ROBERTS, 2001). These antibiotics have been widely used in the prevention and treatment of poultry colibacillosis. However, the long-term use of this antimicrobial family selects for drug resistance in poultry *E. coli* isolates (ZHANG et al., 2012). The major mechanism responsible for tetracycline resistance in enterobacteriaceae is the acquisition of new genes (*tet* resistance genes), which produce new proteins associated with conjugative and/or mobilizable plasmids and transposons (ROBERTS, 2012). So far 43 different tetracycline resistance genes have been found in different bacteria. Twenty-seven of these genes code for energy-dependent efflux proteins, 12 genes code for ribosomal protection proteins, 3 genes code for inactivating enzymes, and 1 gene has an unidentified mechanism of resistance. Twelve of these genes have been found in the *Escherichia* genus (ROBERTS, 2012).

Some of the tetracycline family of antibiotics, such as tetracycline and oxytetracycline, have been used in the Iranian poultry industry for many years. Therefore, this could lead to high tetracycline resistance in some of the bacteria originated from poultry samples in Iran (ABDI-HACHESOO et al., 2014; FALSAFI et al., 2009; RAHIMI et al., 2010). However, no recent data are available on the distribution of tetracycline resistance genes in *E. coli* isolated from broiler chickens in Iran and changes during different stages of the rearing period. The purpose of this study was to evaluate of the tetracycline resistance rate and assess the frequency and alteration of four *tet* resistance genes (*tet*A, *tet*M, *tet*O and *tet*S) during the rearing period of broiler chickens.

Materials and methods

Sampling. Samples were collected from 20 broiler chicken farms located in Shiraz county in the Fars province, Iran. The first stage of sampling was done on one-day-old broiler chicks on their arrival at the farms. The second and the third stages of sampling were done at 30 days of age and one day before slaughter, respectively. At each stage, fifteen cloacal samples were taken separately from the farms using sterile wood applicators. All 3 swabs were pooled in a sterile tube containing tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and immediately transferred to the laboratory.

Isolation and identification of E. coli. Five pooled specimens from each farm were spread onto MacConkey agar (Merck, Germany) plates and incubated overnight at 37 °C. One of the pink color colonies from each plate was picked and spread onto Eosin Methylene Blue (EMB) agar (Merck, Germany) plates and incubated overnight at 37 °C.

Blue-black colonies with dark centers and greenish metallic sheen were considered as *E. coli* and were identified using a panel of biochemical tests (gram stain, oxidase test, TSI test, indole test, citrate test, methyl red and Voges-Proskauer tests and urea agar test) and stored at -70˚C in Tryptic soy broth (TSB) with 30% glycerol until used in this study.

Determination of the tetracycline susceptibility of E. coli isolates. Escherichia coli isolates were tested for tetracycline susceptibility on Mueller-Hinton agar (Merck, Germany) by the disc diffusion method, as described in the National Committee for Clinical and Laboratory Standards (CLSI 2013). The concentration of the tetracycline disks was 30 µg. Tetracycline susceptibility (≥) and resistance (≤) breakpoints (mm) were 19 and 14, respectively (CLSI 2013). Quality control was performed using the *E. coli* ATCC 25922 reference strain.

PCR amplification of tetracycline resistance genes. DNA templates were prepared using the boiling method, as previously described, with some modifications (GREEN and SAMBROOK, 2012). Four tetracycline resistance genes (*tet*(A), *tet*(M), *tet*(O) and *tet*(S)) were investigated among 120 tetracycline resistant E. *coli* isolates (40 isolates for each stage) using PCR. The characteristics of resistance genes, primers and annealing temperatures are given in Table 1. The PCR reaction (20 µL) was performed in 10 mM Tris-HCl, pH = 8.3-8.8, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 10 pmoL of forward and reverse primers (CinnaGen Inc., Tehran, Iran) for each *tet* gene listed in Table 1, and 2 U Taq DNA polymerase (Fermentas; Glen Burnie, Maryland), using 3 µL of the extracted DNA as a template. The final volume of the reaction mixture was adjusted to 20 µL using distilled deionized water. PCR conditions were as follows: (i) 5 min at 94 °C and (ii) 40 cycles, with 1 cycle consisting of 45 s at 94 °C, 1 min at 56-60 °C, 1 min at 72 °C, and (iii) 10 min at 72 °C. *Aeromonas sobria* strain CW4 (JN806155), *Lactococcus garvieae* strain Ira.1s (JN998084), *Pseudomonas putida* strain Fars 110 (JN937120) and *Campylobacter jejuni* strain Shiraz2 (JX853722) were used as positive controls for *tet*(M), *tet*(S), *tet*(A) and *tet*(O), respectively.

Table 1. Characteristics of genes, primers, and annealing temperatures used for detection of tetracycline resistance genes

Gene	Primer name	Primer sequence	Product size (bp)	References
tet(A)	*Tet(A)-F*	5'- GTGAAACCCAACATACCCC-3'	888	(Maynard et al., 2003)
	Tet(A)-R	5'-GAAGGCAAGCAGGATGTAG-3'		
tet(M)	*Tet(M)-F*	5'-GTG GAC AAA GGT ACA ACG AG-3'	406	(Ng et al., 2001)
	Tet(M)-R	5'-CGG TAA AGT TCG TCA CAC AC-3'		
tet(O)	*Tet(O)-F*	5'-AAC TTA GGC ATT CTG GCT CAC-3'	515	(Ng et al., 2001)
	Tet(O)-R	5'-TCC CAC TGT TCC ATA TCG TCA-3'		
tet(S)	*Tet(S)-F*	5'-CAT AGA CAA GCC GTT GAC C-3'	667	(Ng et al., 2001)
	Tet(S)-R	5'-ATG TTT TTG GAA CGC CAG AG-3'		

Statistical analysis. SPSS software (version 16) was used for statistical analysis. Pearson's chi-square test was used to compare tetracycline resistance status and tetracycline resistance gene frequency at the three sampling stages for *E. coli* isolates. Statistical significance was considered to have been achieved when the P value was less than 0.05.

Results

The frequency of *E. coli* resistance to tetracycline varied from 67% in one-day-old chicks to 94% in the chickens ready for slaughter (Table 2). Tetracycline resistance was significantly lower in *E. coli* isolated from one-day-old chicks compared to later sampling stages ($\chi 2 = 31.076$, df = 2, P = 0.001).

Table 2. Antimicrobial resistance rate (%) against tetracycline in 300 *E. coli* isolated from broiler chickens in three stages of rearing period

Stages	Resistance rate (%)		
	Resistance	Intermediate	Sensitive
One-day-old chicks	67	0	33
Thirty-day-old chickens	90	3	7
One- day before slaughter	94	4	2

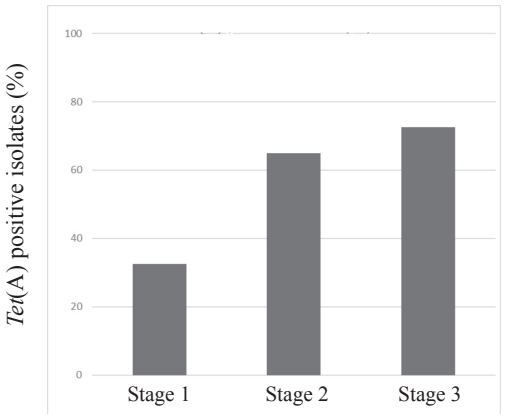

Fig. 1. The frequency of the *tet*(A) resistance gene in 40 tetracycline resistant *E. coli* isolated from different sampling stages (Stage 1: one-day-old, Stage 2: 30 days of age and Stage 3: one day before slaughter)

Of the 120 tetracycline resistant *E. coli* isolates, 68 (59%) carried the *tet*(A) resistance gene. The *tet*(A) resistance gene was present in 32.5% (13/40) of *E. coli* isolated from one-day-old chicks, 65% (26/40) of *E. coli* isolated from thirty-day-old chickens and 72.5% (29/40) of *E. coli* isolated from the chickens on the day before slaughter (Fig. 1). The *tet*(A) resistance gene was significantly lower in tetracycline resistant *E. coli* isolated from one-day-old chicks compared to later sampling stages ($\chi 2 = 14.729$, df = 2, P = 0.001). None of the tested isolates contained *tet*(M), *tet*(O) or *tet*(S).

Discussion

In recent years, *tetracycline* resistance has emerged among many pathogenic and nonpathogenic species of bacteria. This resistance is mainly due to different efflux pump and ribosomal protection genes, mostly associated with mobile components such as plasmids or transposons (ROBERTS, 2012). These pathways of resistance gene acquisition along with the selection pressure hypotheses accelerate tetracycline resistance in commensal and pathogenic bacteria recovered from populated environments, such as poultry farms (FAIRCHILD et al., 2005).

In the present study, *E. coli* isolated from one-day-old chicks showed an unexpected high prevalence of tetracycline resistance, suggesting a high prevalence of tetracycline resistance in the primary intestinal microflora of these broiler chicks. It has been stated that antimicrobial treatments on broiler breeder farms may affect the prevalence of antibiotic resistant isolates in their progenies, without use of antimicrobials on commercial broiler farms (OZAKI et al., 2011). Vertical transmission from broiler breeders, followed by horizontal transmission in hatcheries has been stated as an important approach for distribution of *E. coli* clones on poultry farms (GIOVANARDI et al., 2005).

Interestingly, a significant increase in the number of tetracycline resistance *E. coli* isolates was found on the farms in our study during the 2nd and 3rd stages of rearing. Early horizontal transmission in hatcheries enables a massive amplification of special *E. coli* clones into a large population of poultry reared on integrated farms (GIOVANARDI et al., 2005). Potential transmission of tetracycline resistance genes via these clones could increase tetracycline resistance

during the rearing period. This resistance could occur with or without selection pressure. However antibiotic consumption accelerates this process (FAIRCHILD et al., 2005). DA COSTA et al. (2010) showed that tetracycline resistance has increased in enterococci isolated from antibiotic medicated and non-medicated growing broilers during the rearing period, so that tetracycline resistance in medicated broilers was 100% on the thirty-third day of their study.

Screening of the tetracycline resistance gene showed that *tet*(A) was the only detected *tet* resistance gene in these *E. coli* isolates. *Tet*(A) belongs to the efflux genes, while

tet(M), *tet*(O) and *tet*(S) are categorized as ribosomal protection genes. The efflux genes are the most commonly found *tet* genes in aerobic and facultative gram-negative bacteria (ROBERTS, 2012). Although the *tet*(A) gene has also been reported to be the predominant *tet* resistance gene in poultry *E. coli* isolates (ANDERSON et al., 2014; BRYAN et al., 2004; SENGELØV et al., 2003; SMITH et al., 2007; ZHANG et al., 2012), it was still notable that, in this study, none of the *E. coli* isolates carried *tet*(M), *tet*(O) and *tet*(S). Unexpectedly, in a noticeable number of resistant isolates (n = 52), no resistance genes were detected. This situation may be related to isolates that contain other tetracycline resistance genes, or other resistance mechanisms (ROBERTS, 2012).

During the rearing period of broilers, trends in *tet*(A) resistance gene distribution in tetracycline resistant *E. coli* isolates showed that the percentage of this gene in 2nd and 3rd stages was remarkably higher than at the earlier stage of sampling. The *tet*(A) gene is commonly associated with plasmids, and can be easily transferred between gram negative bacteria (ANDERSON et al., 2014; ROBERTS, 2012). Similarly, an experimental farm study has demonstrated a significant decrease in *tet*(B) carriage and a significant increase in *tet*(A) carriage after tetracycline administration (SMITH et al., 2007). SENGELØV et al. (2003) showed that the *tet*(A) resistance gene was significantly higher in pathogenic *E. coli* isolated from broilers, while other *tet* resistance genes were higher in non-pathogenic *E. coli* in comparison with pathogenic *E. coli*. The high prevalence of *tet*(A) gene in *E. coli* isolates from the later stages of the rearing period may be due to selective pressure, as well as the high transferability of the *tet*(A) determinant (CHOPRA and ROBERTS, 2001; FALSAFI et al., 2009).

Rising trends of tetracycline resistance and the *tet*(A) resistance gene distribution during the rearing of chickens in broiler farms in this area may be worrying. Tetracycline, oxytetracycline and doxycycline are among the most common antibiotics on poultry farms in this region, used by farmers without prescriptions (personal communication). Our study on tetracycline resistant isolates of avian *E. coli* may be used as a guide for clinical medication and prevention using this family of antibiotics in this area. Our results also raise the possibility of tetracycline resistance gene dissemination to other pathogenic and commensal bacteria in the poultry industry, especially in the later stages of broiler rearing.

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SAŽETAK

Istražena je otpornost na tetraciklin izolata vrste *E. coli* (n = 300) izdvojenih iz tovnih pilića triju dobnih skupina (jednodnevnih, 30-dnevnih i pilića jedan dan prije klanja). Ukupno je 120 izolata *E. coli* otpornih na tetraciklin bilo pretraženo na prisutnost gena za otpornost na tetraciklin (*tet*(A), *tet*(M), *tet*(O) i *tet*(S)). Otpornost na tetraciklin u izolatima iz jednodnevnih pilića iznosila je 67%, u izolatima iz 30-dnevnih pilića 90%, a u onih izdvojenih iz pilića dan prije klanja 94%. Od 120 izolata otpornih na tetraciklin, 68 (59%) nosilo je gen *tet*(A). Gen *tet*(A) dokazan je u 32,5% (13/40) izolata *E. coli* iz jednodnevnih pilića, u 65% (26/40) iz 30-dnevnih pilića i 72,5% (29/40) iz pilića dan prije klanja. Nijedan od pretraženih izolata nije sadržavao *tet*(M), *tet*(O) or *tet*(S). Otpornost na tetraciklin bila je relativno visoka u izolata iz jednodnevnih pilića, što upućuje na zaključak o visokoj prevalenciji otpornosti primarne crijevne flore na tetraciklin. Značajno povećanje stope otpornosti izolata *E. coli* ustanovljeno je tijekom 2. i 3. uzgojnog razdoblja. *Tet*(A) bio je jedini dokazan *tet* gen u pretraženih izolata. Povećana učestalost gena otpornosti *tet*(A) tijekom uzgojnog razdoblja tovnih pilića je zabrinjavajuća jer se plazmid prenositelj gena *tet* može prenijeti na druge patogene i komenzalne bakterije u peradi.

Ključne riječi: *Escherichia coli*, otpornost na tetraciklin, *tet*(A), tovni pilići